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The antibacterial activity of blue light against nosocomial wound pathogens growing planktonically and as mature biofilms

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Running Head: Activity of 400 nm light against nosocomial pathogens

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ABSTRACT

Background

The blue wavelengths within the visible light spectrum are intrinisically antimicrobial, and can photodynamically inactivate the cells of a wide spectrum of bacteria (Gram-positive and -negative) and fungi. Furthermore, blue light is equally effective against both drug sensitive and resistant members of target species, and (in contrast to UV radiation), is less detrimental to mammalian cells.

Blue light is currently used for treating acnes vulgaris, and *Helicobacter pylori* infections; the utility for decontamination and treatment of wound infections is in its infancy. Furthermore, limited studies have been performed on bacterial biofilms; the key growth mode of bacteria involved in clinical infections.

Here we report the findings of a multicentre *in vitro* study performed to assess the antimicrobial activity of 400 nm blue light against bacteria in both planktonic and biofilm growth modes.

Methods

Blue light was tested against a panel of 34 bacterial isolates (clinical and type strains) comprising: *Acinetobacter baumannii*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus, Enterococcus faecium*, *Klebsiella pneumoniae*, and *Elizabethkingia meningoseptica.*

Results

All planktonic phase bacteria were susceptible to blue light treatment, with the majority (71%) demonstrating a ≥5 $\log_{10}$ decrease in viability after 15-30 minutes exposure (54 J/cm^2 to 108 J/cm^2). Bacterial biofilms were also highly susceptible to blue light, with significant reduction in seeding observed for all isolates at all levels of exposure.

Conclusions

These results warrant further investigation of blue light as a novel decontamination strategy for the nosocomial environment, as well as additional wider decontamination applications.

Importance

Blue light shows great promise as a novel decontamination strategy for the nosocomial environment, as well as additional wider decontamination applications (e.g. wound closure during surgery). This warrants further investigation.

1 INTRODUCTION

Antimicrobial resistance (AMR) is rapidly evolving and emerging to be a large threat to modern medicine. Although only affecting a minority of admissions, healthcare associated infections are associated with increased mortality, prolonged hospital stays and increased treatment costs (1). With the rise in resistance to the carbapenem class of antibiotics in Gram-negative organisms (2), there is a significant threat of infections becoming wholly untreatable with current treatment regimens (3,4).

Much research is now focussed on alternatives to the conventional antimicrobial agents. These mostly involve topical agents (with the aim to reduce surface contamination and therefore lower the risks of sepsis and infection progression) with research to date on a large number of agents. Since the environment is a key source of nosocomial pathogens (5), there has also been renewed focus on hospital cleaning and disinfection, especially *via* antimicrobial chemicals delivered in a novel way, including antimicrobial light sources (1,6). These novel strategies capable of decontaminating both the patient's wound and the environment, offer to be highly beneficial in the fight against AMR and nosocomial infections.

The blue wavelengths within the visible light spectrum (especially wavelengths between 400 nm to 470 nm) are intrinsically antimicrobial and do not require additional exogenous photosensitizers to exert an antimicrobial effect (4). Photodynamic inactivation of both bacterial and fungal cells occurs as a result of photo-excitation of intracellular porphyrins (1) by blue light, leading to energy transfer and the production of highly cytotoxic reactive oxygen species (ROS); primarily singlet oxygen ($^1O_2$) (4, 7-9). All wavelengths from 400-425 nm can be used for microbial inactivation; however the optimal antimicrobial activity occurs at 405 nm, since this is the point in the electromagnetic spectra where maximum porphyrin excitation occurs (10). Although less germicidal compared to ultra-violet light (1), pathogens can be selectively inactivated without damaging human cells and consequently blue light is considered much less detrimental to mammalian cells than ultra-violet (11,12). One potential benefit of light-based antimicrobial therapies is an equal efficacy against drug sensitive and resitant members of target species (13,14).

Blue light has been shown to exhibit a broad spectrum of antimicrobial effect against bacteria and fungi, although, generally the Gram-positive bacteria are considered to be more susceptible to blue light than the Gram-negatives (15,16). Successful inactivation has been demonstrated *in vitro* against *Staphylococcus aureus* (including MRSA), *Clostridium difficile* (both spores and vegetative cells)*, Acinetobacter baumannii, Escherichia coli, S. epidermidis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Streptococcus pyogenes,* and *Mycobacterium* spp. (14-15, 17, 18). In addition to the key nosocomial pathogens, blue light is also effective against *Propionibacterium acnes*, and has been used topically to treat acne vulgaris (19, 20), and *Helicobacter pylori*, where blue light is used internally as a 'light string' to treat stomach infections (21). Owing to the mechanism of action of blue light, it is unlikely that viruses will be susceptible unless photosensitizers are added to enhance virucidal activity (22).

The use of blue light for treatment of wound infections *in vivo* is an emerging technology. To date blue light therapy has been shown to significantly reduce the bacterial burden of wounds infected with *P. aeruginosa* (23), MRSA (24), and *A. baumannii* (25), and saved the lives of mice subjected to potentially lethal burns contaminated with *P. aeruginosa* and *A. baumannii* (23, 25).

As well as clinical application for patient treatment, blue light is also a promising candidate for the control of problematic microorganisms in the clinical setting (e.g. the disinfection of air and exposed surfaces). In this regard, Bache *et al* (26) and Maclean *et al* (1) have performed studies with a new disinfection technology termed the HINS-light environmental decontamination system (EDS) which delivers low-irradiance 405nm light continuously and is suitable for use in patient occupied settings. Evaluation studies performed by the latter authors showed that there was a statistically significant 90% reduction in numbers of culturable *Staphylococci* spp. following 24 hours of use in an unoccupied room (5), and reductions of 56-86% when used in burns isolation rooms occupied by MRSA-positive patients. Furthermore, when the system was no longer used, the room became recontaminated to levels similar to those pre-treatment.

The vast majority of research on blue light has been carried out on bacteria in their planktonic phase, dispersed evenly in a liquid medium. In nature this is rarely the case, since most bacteria aggregate to form complex communities within a matrix of extracellular polymeric substances termed a biofilm. There are many advantages for this compared to planktonic growth which include: increased resistance to killing *via* antimicrobials, immune cells, chemicals and environmental stresses (27). Furthermore, once a biofilm has become established on a surface they are extremely hard to eradicate. Medically, biofilms have been associated with a myriad of chronic infections, acute infections, colonisation of in-dwelling medical devices, and wound infections (27-29).

Since we know that the majority of clinical infections and environmental contamination involve microbial biofilms (30), this multi-centre *in vitro* study was performed to assess

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the antibacterial activity of blue light against biofilms of a range of important nosocomial pathogens.

2 MATERIALS AND METHODS

A series of *in vitro* experiments were conducted with a panel of organisms (Table 1) to determine the efficacy of blue light (400 nm) against bacteria in a planktonic (free-floating in broth) and biofilm (attached to a surface) mode of growth. The panel comprised well-characterised control and clinical isolates (in terms of their antibiogram and ability to form biofilms *in vitro*) and concentrated mostly on *A. baumannii* strains from a protracted outbreak at the Queen Elizabeth Hospital in Birmingham (QEHB) (31). ***A**. baumannii* is a key nosocomial pathogen which survives in hospital and healthcare environments despite conditions such as desiccation, nutrient starvation and antimicrobial chemicals (e.g. disinfectants) (332, 33). Despite stringent infection control practices, a large outbreak of *A.baumannii* occurred at QEHB where 65 patients tested positive during the outbreak period (July 2011 to February 2013). The strains from this outbreak demonstrated a high degree of resilience to survival in the hospital environment and there was also evolution amongst the isolates over time to increase dessication resistance and biofilm formation capacity. Additional *A. baumannii* isolates (representing genetically diverse strains) were tested in this panel to add some diversity to the strains, including strains ACI_AYE (a representative of International Clone I; a major globally relevant lineage), ACI_C60 (a control strain of a unique PFGE type), and ACI_19606 (a control strain of a further unique PFGE type) (typing data not shown).

We also tested a small range of other 'comparator' organisms commonly causing hospital acquired infection including *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *P. aeruginosa*, *E. coli, S. aureu*s*,* and *Enterococcus faecium* and included control strains (PS_6749 and MSSA_10788) recognised in the EN standards for assessing the efficacy of chemical disinfectants (e.g. EN 13727 (34)). The panel comprised isolates that in previous tests had demonstrated ability to form relevant quantitities of biofilm *in vitro*, and furthermore included two carbapenem (multi-drug) resistant isolates of *K. pneumoniae*, and a single isolate of *Elizabethkingia meningoseptica* from a wound swab. This is an intrinsically highly resistant organism, usually resistant to extended-spectrum ß-lactam agents (due to production by most strains of two betalactamases: one ESBL and one Class B carbapenem-hydrolyzing metallolactamase), aminogylcosides, tetracycline, and chloramphenicol (35).

All isolates were stored at -80°C on Protect™ beads, and were routinely cultured on cysteine lactose electrolyte deficient (CLED) or blood agar prior to each experiment.

Experiments were designed to assess the antibacterial activity of blue lightagainst planktonic and biofilm growth forms of the panel of bacteria described above. Testing was performed at the Defence Science Technology Laboratory (DSTL) (planktonic) and the Surgical Reconstruction and Microbiology Research Centre (biofilms), and blue light of 400 nm wavelength was used for all experiments.

2.1 Blue light equipment

High intensity blue light was provided by a LED Flood array (Henkel-Loctite, Hemel Hempstead, UK). This array utilises 144 reflectorized LEDs which produce a homogeneous illuminated area of 10 cm x 10 cm. The emission spectrum of the LED array was determined using a USB2000 spectrophotometer (Ocean Optics, Oxford, UK). Two identical platforms were used for the testing, both of which were calibrated at DSTL using a PM100D radiant power meter (Thorlabs, Newton, New Jersey, USA) prior to *in vitro* testing to ensure a reproducible irradiance of 60 mW/cm^2 when the LED array is positioned 15.5cm above the test area. All of the experimental conditions (except wavelength) adhere to the optimal criteria outlined by Coohill and Sagripanti (36) for the assessment of bacterial sensitivity to UV-C radiation.

2.2 Impact of blue light on planktonic bacteria

Bacterial isolates were grown overnight in Luria Broth (LB) [Sigma-Aldrich, UK], then diluted in sterile PBS to produce a starting concentration of approximately 1×10^6 bacteria per ml. Test samples (2ml) were inoculated into a 12-well microtitre plate [Corning, New York, USA], sealed with an optically clear ABsolute qPCR sealer [Thermo Fisher Scientific, Paisley, Scotland] to prevent evaporation, then exposed to blue light for 30 minutes (samples were taken for viable counting at 5 minute intervals). If the strains still showed viability after 30 minutes of blue light exposure, the test was repeated over 180 minutes, with samples taken at 20 minute intervals. An identical dark control plate was set up, wrapped in aluminium foil and placed in the flood array adjacent to the blue light irradiated samples.

At time increments during the experiment blue light exposed and dark incubated samples were removed and viable bacteria enumerated by serial dilution and growth on LB agar plates. The blue light sensitivity for each strain was determined from the mean of three independent biological replicates, with two technical replicates within each experiment.

The blue light dose (J/cm^2) received by the bacteria was calculated by multiplying the irradiance of light (W/cm^2) to which the sample was exposed, by the exposure time (seconds).

2.3 Impact of blue light on pre-formed biofilms

The antibacterial activity of blue light against pre-formed biofilms was assessed by conducting 'minimum biofilm eradication concentration' (MBEC) experiments (37) on each isolate. Overnight LB cultures of the test strains (made by inoculating approximately three to five colonies into 5ml of fresh LB broth and incubating at 37°C overnight) were diluted in fresh antibiotic-free Mueller-Hinton (MH) broth to an OD_{600} of 0.1 and then 200µl seeded into wells of a 96-well microtiter tray (MTT). Positive (200µl 0.1 OD_{600} diluted organisms) and negative (200µl MH broth) controls were included per blue light time point to be tested.

To produce a 'transferable biofilm', a 96 well polypropylene plate [Starlabs, UK] was then placed into the MTT so that each well contained a 'peg', on which biofilms could form, before the plates were sealed, and statically incubated at 33°C for 72 hours. After 72 hours, the pegs (±biofilm) were removed and washed in a MTT containing sterile water (to remove any unbound cells). The positive (bacteria only) and negative control (sterile broth only) 'peg plate' was placed in a clean, empty MTT and wrapped in foil. Following this, both the control and the test peg plate were placed in the test area (15.5.cm beneath the light source) and exposed to the blue light for time points of 15, 30, 45 or 60 minutes (corresponding to a blue light dose of 54, 108, 162 and 216 J/cm^2 respectively). The foil around the control plate prevented the pegs from receiving any blue light treatment (and hence these positive control biofilms were not exposed to the blue light), but the control plate biofilms would have most likely been exposed to the same amount of heating and drying as the blue light exposed test plate.

After the treatment, the peg plates were carefully placed into a MTT containing 200µl sterile MH broth (herein referred to as 'reporter broth') for overnight incubation. After 18 hours, the OD of the reporter broth was measured to assess the viability (seeding) of the biofilms following blue light exposure.

To demonstrate the presence of biofilms on the pegs, crystal violet (CV) assays were additionally performed on the pegs after the OD of the reporter broth had been measured. This involved placing the pegs into MTTs containing 200µl of 1% CV (which binds to any present microbial biomass of biofilm), followed by washing (to remove unbound CV) and subsequent solubilisation of the CV in 200µl of 70% ethanol. The peg biofilm biomass could then be measured using OD readings as previously and the presence of the biofilm confirmed. Two biological and 10 technical replicates were performed for each strain and blue light exposure duration, respectively.

2.4 Statistical analysis

2.4.1 Planktonic tests

For the planktonic data, the surviving fraction was determined from the quotient N/N_0, with N = the number of colony formers of the irradiated sample and N_0 that of the non-irradiated controls. Plotting the logarithm of N/N_0 as a function of dose (blue light fluence in J/cm²), allowed survival curves to be obtained.

To determine the curve parameters, the following relationship was used: $\ln N/N_0 = IC \times F + n$ where: N = the number of colony formers after blue light irradiation; N_0 = the number of colony formers without irradiation; IC = inactivation constant (cm^2/J); and n = extrapolation number, (i.e. the intercept with the ordinate of the extrapolated semi-log straight-line). The inactivation constant and the reciprocal lethal dose (LD) values were determined from the slope of the dose–effect-curves (linear portion of the curve).

To allow for comparison with other bactericidal radiation sources in the literature, blue light mediated killing was calculated in terms of the inactivation constant slope (IC), and the kill kinetics shown as both the LD_{37} and LD_{90}. The significance of the difference of the dose–effect-curves was statistically analyzed using student's t-test. Differences with P values <0.05 were considered statistically significant.

LD_{90} values were analysed using the statistical software package IBM SPSS V21.0, were found to be log normal by QQ plot (data not shown), and were consequently transformed to the logarithm of 10 prior to parametric analysis. Differences between bacterial species was investigated using a 1 way ANOVA, and the suitability of the data for parametric analysis was further established with the use of a Levene's test for unequal variance (P=0.165). Where only one bacterial strain of species was available, this species was taken out of the analysis. Multiple comparisons were made using the Bonferroni's correction. Similarly, the effect of pigmentation of *S. aureus* strains on susceptibility to blue light was tested by using T-tests without Welches correction and suitability was further tested using Levene's test for unequal variance (P=0.984).

2.4.2 Biofilm tests

The ability of biofilms to seed new growth following exposure to blue light was assessed by comparing the OD values at each blue light time point verses the untreated (positive) control, and significance was determined using the student's t test. In order to investigate any possible link between biofilm size/depth (colourimetry), and blue light sensitivity, these two parameters were investigated through QQ plots in SPSS (version 21.0; SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Initial analysis suggested a transformation of both parameters by the logarithm of 10 was needed to render the data suitable for parametric analysis (analysis not shown). Very little difference between the technical replicates was observed with regards to either parameter and therefore the median of the $\log_{10}$ of the technical replicates was used for analysis. The capacity for each strain to form a biofilm was taken from the average of the OD values over the 4 time points for the positive control. Comparisons of biofilm values were made using 1 way ANOVA and students' 't' tests (without Welch's correction) and comparisons of variances were made with F tests and Brown-Forsythe tests. The viability of each strain of bacteria in biofilm was analysed by Bonferroni's posts tests across each time point (SPSS). Where significant differences between the positive control and the blue light occurred, the blue light was regarded as having an effect from there on leading to an ordinal score for each strain of 15 min (54 J/cm^2), 30 min (108 J/cm^2), 45 min (162 J/cm^2), 60 min (216 J/cm^2), or >60 min. Comparisons of biofilm sensitive scores were made using Kruskal-Wallis tests.

In order to characterise whether correlation existed between measured parameters, the Spearman's method was used. In order to determine the statistical power of the correlations, the computer program SPSS sample power V3.0 (IBM) was used and power was calculated for one sample correlations using the derived R value and the sample size (N = 34).

3 RESULTS

Blue light was tested against 34 bacterial isolates; including clinical isolates from QEHB and culture collection type strains. The results of the spectral output testing of the blue light platform (with an Ocean Optics USB2000 spectrometer), determined the emission peak of the blue light produced was at 400nm, with a full-width half maximum value of ±8.5 nm (Fig. S1).

3.1 Sensitivity of isolates to blue light when grown in planktonic culture

All 34 isolates were sensitive to blue light treatment, and there was no significant decay in the dark incubated controls. In contrast, rapid and substantive loss of viability was observed where all test bacteria were exposed to blue light (Figure 1A-F).

Twenty four of the isolates (71%) demonstrated at least a 5 $\log_{10}$ decrease in viability following 15 (54 J/cm^2) to 30 minutes (108 J/cm^2) of blue light exposure (Table 2), and for the majority of these isolates (*A. baumannii* (12/12), *S. aureus* (4/5), *S. maltophilia* (2/3), *E. meningoseptica* (1/1)), there was a greater than 6 $\log_{10}$ decrease in viability. Ten of the 34 isolates showed <5 $\log_{10}$ decrease in viability. The isolates concerned included *E. cloacae* (ENTCL_525, ENTCL_801, ENTCL_804), *E. coli* (EC_073, EC_042), *K. pneumoniae* (MDR_A, MDR_B), *S. aureus* (MSSA_10788), *S. maltophilia* (STEMA_551), and *E. faecium* (EFM_513). Four of the 34 isolates (*E. cloacae* (ENTCL_525, ENTCL_801, ENTCL_804) and *E. faecium* (EFM_513)), took longer to kill than the majority of isolates, requiring extended timepoints up to 120 minutes (432 J/cm^2) to obtain 2-3 $\log_{10}$ decrease in viability.

Loss of bacterial viability associated with blue light was calculated as previously described to give LD_{90} values in terms of J/cm^2. Investigation of these LD_{90} values indicated that differences between LD_{90} values were very likely driven by differences in the blue light susceptibility of different bacterial species ($P<0.001$). We found that the highest LD_{90} values belonged to *E. cloacae* and *K. pneumoniae* strains which had values statistically higher than all other species included in the analysis (where more than one representative strain was tested) ($P<0.05$ in all cases) (Figure 2). The exception to this was *E. coli* which had moderate blue light tolerance, but no statistical differences were seen between the strains tested . *A. baumannii*, *S. aureus*, *P. aeruginosa* and *S. maltophilia* all had similar and low levels of resistance to the blue light exposure.

In the initial assay of planktonic cell resistance to blue light, we observed that the *S. aureus* strains demonstrated different colony pigmentation; appearing either as pale yellow (MRSA_508, MRSA_520, MRSA_531 and MSSA_F77) or orange (MSSA_10788) when grown on LB agar. We hypothesised that this pigmentation may be responsible for the variability seen in both the survival fraction curves and LD_{90} values when exposed to blue light (Figures 1B and 2). Four additional culture collection strains of *S. aureus* were assessed for blue light sensitivity, including two pale yellow (MSSA_29213, MSSA_10442) and two orange (MSSA_33807, MSSA_4163) strains. In total nine strains of *S. aureus* were tested, six yellow and three orange. We determined that the orange pigmentation correlated with increased resistance to blue light in both the survival fraction curves and in LD_{90} values (Figures 3A and B). The LD_{90} values were statistically significantly higher in the orange pigmented strains than in their yellow counterparts ($P=0.003$).

3.2 Sensitivity of isolates to blue light when grown in biofilms

Blue light treatment resulted in reductions in biofilm seeding for all isolates tested (Figure 4A-C), and the majority of these reductions (apart from timepoint 15 minutes for MSSA_10788) were statistically significant (p-values <0.05 in student's t-tests compared to the positive control). The percentage reductions are shown in table 3 with the single non-significant result denoted by a ^.

The most susceptible isolates were the Gram negative organisms, and in particular ACI_19606, where there was a 93.5% reduction in biofilm seeding ($p<0.001$) after 15 minutes (54 J/cm^2) of blue light exposure. As a group, the other Gram negative comparator organisms were the most susceptible, with 10/16 (63%) showing greater than 80% reductions in biofilm seeding (average = 86%) at 15 minutes, compared to 1/12 for *A. baumannii* and 1/6 for the Gram positive organisms. Although ENTCL_804 responded well to blue light treatment at 30 minutes/108 J/cm^2 (46.6% reduction), 45 minutes/162 J/cm^2 (88.2% reduction), and 60 minutes/216 J/cm^2 (87.8% reduction), the treatment actually resulted in increased biofilm seeding at 15 minutes of 18.7%. This result was repeatable and was seen in a number of replicates.

As mentioned, the Gram positive biofilms were less susceptible to blue light treatment, with only two isolates (33%) achieving at least 90% reductions in seeding. It is important to note however the small sample size. One isolate of *S. aureus* (MSSA_10788), which is recognised in the EN standards for assessing the efficacy of chemical disinfectants was the least sensitive to blue light, achieving a maximum reduction in biofilm seeding of 36% at 45 minutes (162 J/cm^2). This result was again repeatable and was seen in 48replicate pegs. This is further evidence towards the hypothesis that bacterial pigmentation attenuates the sensitivitiy to blue light, in biofilms as well as in planktonic cells.

In order to characterise how the different biofilm forming properties (seeding ability and biofilm size) of each bacterial species relate to each other, a series of correlations were performed. We found no evidence for significant correlations existing between i) median biofilm size (CV assay) and median sensitivity of biofilm to blue light (P = 0.133), or ii) median biofilm size and LD_{90} (P = 0.912). For these reasons we feel that any differences between species in biofilm resistance to blue light are likely to be intrinsic differences rather than a function of the biofilm. However, we are not able to dismiss the alternative hypothesis that correlations do exist as these analyses were insufficiently powered. We found the statistical power to be 21% when considering the potential correlation between planktonic and biofilm killing (R = 0.200) and 33% when considering the correlation between biofilm killing and biofilm formation (R = -0.263). In this respect, we must actually conclude that real correlations between these parameters might exist; however, if they do exist, they are likely to less apparent in comparison to the correlation observed between biofilm formation and planktonic killing.

We found a significant correlation between the sensitivity of strains to blue light in the planktonic state and their ability to form biofilms (Spearman's Coefficient = 0.369, P = 0.032). This indicated that strains that demonstrated greater resistance to blue light in planktonic state were more likely to produce thicker biofilm.

4 DISCUSSION

In this study, we have shown blue light (400 nm) to be effective at inactivating both planktonic cells and biofilms of important nosocomial wound pathogens. Contrary to published research (1,15,16), we found Gram negative organisms to be more susceptible to blue light. There are a number of differences between these published studies and our study which may contribute to these conflicting findings. Firstly, we tested a number of isolates per species (most of the studies test one strain of each species) comprising both clinical and control strains (most of the published studies use control strains which may have been passaged many times), and our light box exposed the bacteria to higher doses (60mW/cm^2) than the 10mW/cm^2 used by Maclean et al (15). Although we only tested a small number of isolates, the Gram positive biofilms appeared less sensitive to blue light treatment, with one strain (MSSA_10788) consistently resisting the effects of blue light.

Analyzing blue light susceptibility against multiple clinical strains from the same species has permitted us to assess the heterogeneity of intra-species kill rates. In some species such as *A. baumannii* the rate of blue light mediated killing was extremely homogeneous; however, *S. aureus* strains display a much more heterogeneous response to blue light stress.

It has long been recognised that bacterial cells have utilised pigmentation as a virulence factor (38). One of the most easily recognisable bacterial pigments are the triterpenoid carotenoids, which impart the eponymous golden colour to *S. aureus* strains. Various authors have identified a correlation between strains containing the carotenoid pigment staphyloxanthin and the ability to survive on surfaces exposed to natural sunlight (39), and it is well known that carotenoids function as antioxidants. Furthermore, staphyloxanthin has been shown to provide protection for pigmented *S. aureus* strains against ROS produced by phagocytes (40, 41).

Augmentation of the clinical isolates of *S. aureus* with a series of well characterized strains from culture collections allowed us to correlate increased blue light killing times (as seen with MSSA_10788) with colony pigmentation. Planktonic testing and assessment of the LD_{90} values per colour group, show that the light sensitivity of the strongly orange pigmented strains is significantly different to the standard pale yellow strains (p-value <0.003) (Figure 3B). Therefore, although all species of *S. aureus* tested were susceptible to blue light, it is important to consider the effects of bacterial pigmentation when determining the required blue light exposure for effective decontamination.

As well as differences in sensitivity, there were also several instances where blue light treatment increased the planktonic growth and biofilm seeding. For example, with ENTCL_804, there was an increase of 18.7% in seeding after 15 minutes of blue light treatment. Light has been shown to facilitate growth when the wavelengths and dose are not appropriate (42), and Nussbaum *et al* (43) found that 810 nm and 905 nm improved the growth of *E. coli* and *S. aureus*, respectively. Furthermore, Mussi et al (44) reported that blue light treatment decreased motility and biofilm formation in *A. baumannii*, and increased pathogensis when co-cultured with *Candida albicans* (a model for apoptosis in human alveolar macrophages) (45). However, there are several important differences between studies; the fluence was considerably lower than in this study (1.32-1.89 mW/cm^2 verses 60 mW/cm^2) and the light wavelength peaked at 460 nm verses 400 nm, while the exposure was measured over 4 days verses 30 minutes. However, it does raise interesting questions on the effects of suboptimal light exposure on bacterial cells which should be looked into in future studies. Furthermore, the enhanced growth in our study warrants further investigation.

We found that, a correlation existed between the strains which were sensitive to blue light in planktonic state and those which produced larger amounts of biofilm in the CV assay. The reason for this observation is not clear in this investigation; however we hypothesise that this indicates that blue light selective pressure may exist in environmental niches where protection as a biofilm might provide benefits against other stimuli that are likely to co-exist with blue light. The fact that we were unable to observe a correlation between biofilm and planktonic resistance to blue light might indicate that these mechanisms are functionally independent. Planktonic cells need to rely on their intrinsic transparency, pigmentation and repair to protect against blue light. In biofilm bacteria can rely on more extracellular exudate and neighbours to protect against blue light.

To the best of our knowledge, our work is one of the first to show the antibacterial activity of 400 nm light against a range of clinically relevant bacterial strains (as well as control strains), and one of just a handful to look at non-dental biofilms. The inclusion of multiple isolates is an additional strength of the paper as it allows correlations between phenotypic characteristics and blue light resistance to be explored. Although there are several limitations (monomicrobial biofilms tested instead of polymicrobial, no formal assessment of the potential for the development for resistance, the relatively small number of isolates tested, and the potential biasing effect of the included resistant isolates), our work nonetheless provides valuable insights into this technology, and especially how it relates to the eradication of biofilms for environmental decontamination.

The findings in this paper demonstrate that high intensity blue light can be used to inactivate a wide range of clinical pathogens, not only in the planktonic state but also as mature biofilms. This technology has many practical applications within healthcare settings, as blue light may ameliorate opportunistic infections indirectly by reducing the bacterial load on environmental surfaces and directly within wounds. Future studies are warranted to investigate this further, and especially whether the exposure times of the 400 nm blue light can be reduced for a range of different clinical applications. As blue light is equally efficacious against antibiotic resistant pathogens, this technology may prove an important weapon in the future fight against antimicrobial resistance.

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7 Disclaimer

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Figure Legends:

Figure 1. Survival of planktonic bacteria after exposure to 400 nm blue light.

Strains: *A. Acinetobacter baumannii* strains: closed circles (• ACI_616; open circles ACI_618; closed triangles up ACI_AYE; open triangles up ACI_665; closed triangles down ACI_19606; open triangles down ACI_648; closed diamonds ACI_659; open diamonds ACI_C60; closed squares ACI_671; open squares ACI_672; closed hexagons ACI_698; open hexagons ACI_642.

B. Staphylococcus aureus: open circles MSSA _10788; closed triangles up: MSSA_F77; closed squares MRSA_520; open triangles up MRSA_531; closed circles MRSA_508

C. *Stenotrophomonas maltophilia*: circles STEMA_ 558; triangles up STEMA_551; squares STEMA_529

D. *Enterobacter cloacae:* circles ENTCL_804; triangles up ENTCL_801; squares ENTCL_525

E. *Pseudomonas aeruginosa*: closed circles: PSE_1586; open circles PSE_PAO1; closed triangles up PSE_568; closed squares PSE_1054; open squares PSE_6479

F. Other: *E. coli* EC_042 open circles: *E. coli* EC_073 closed circles; *K. pneumoniae* MDR-A open squares: *K. pneumoniae* MDR-B closed squares; *Elizabethkingia meningoseptica* open triangles up EKIN_502; *Enterococcus faecium* EFM_513 open triangles down

Data are averages ± standard deviations (n = 3).

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Figure 2. Comparison of blue light LD_{90} values between strains and species. Each individual circle represents the average LD_{90} for each strain ± standard deviations (*n* = 3). The average LD_{90} value for each species is shown by horizontal lines.

Figure 3. A. Correlation between survival of planktonic *S. aureus* strains following blue light exposure, and cell pigmentation.

Orange carotenoid producing strains: closed circles MSSA_4163; closed triangles down MSSA-33807; closed triangles up MSSA_10788. Yellow non-carotenoid producing strains: open circles MSSA_10442; open squares MSSA_F77; open diamonds MRSA_520; open triangles down MRSA_531; open triangles up MRSA_508; open hexagons MSSA_29213. Data are averages ± standard deviations (*n* = 3).

B. Comparison of blue light LD_{90} values between yellow and orange pigmented *S. aureus* strains.

Each individual circle represents the average LD_{90} for each strain ± standard deviations (*n* = 3). The average LD_{90} value for yellow and orange pigmented strains is shown by horizontal lines.

Figure 4, A-C: Graphs showing the biofilm seeding results for all isolates.

Optical density on the y axis refers to the average biofilm seeding for the isolates tested after exposure to blue light (BL) at the range of durations tested (in minutes) on the x axis. Positive control: refers to the average biofilm seeding of the dark incubated, non blue light exposed isolates. Negative control: refers to a negative (broth only) control. The error bars represent the standard error.

Tables:

Table 1: List of the clinical and control isolates used in this study.

Table 2: Antimicrobial effects of blue light on planktonic cells

Table 3: Average percentage change in biofilm seeding in isolates exposed to blue light compared to non-exposed dark incubated controls

Supplementary:

Figure S1: Emission spectrum of Henkel Loctite blue light array determined using an Ocean Optics USB2000 spectrometer

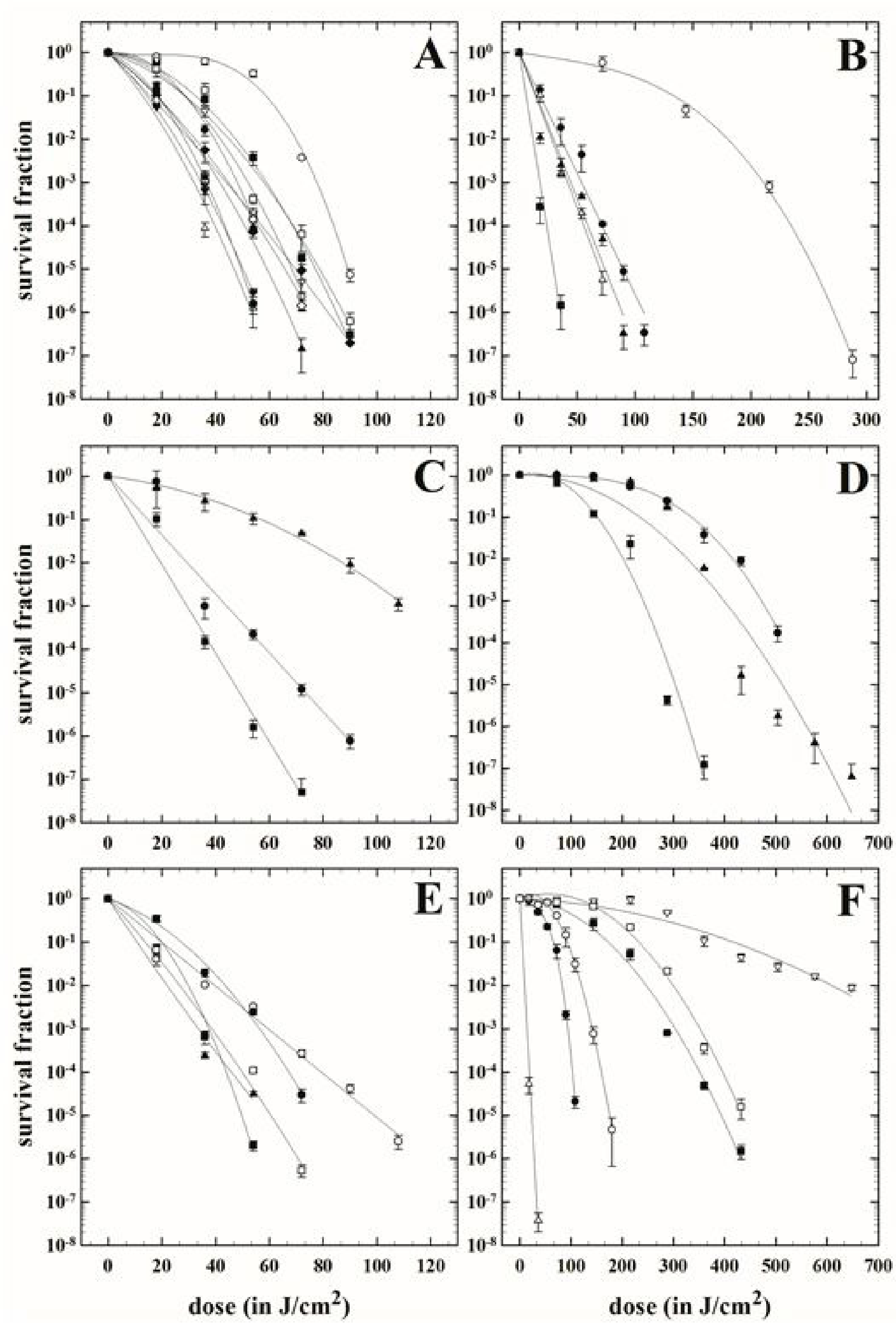

Figure 1. Survival of planktonic bacteria (in terms of survival fraction) after exposure to 400 nm blue light. **A:** *A. baumannii*, **B:** *S. aureus*, **C:** *S. maltophilia*, **D:** *E. cloacae*, ***F:*** Other Gram negative organisms.

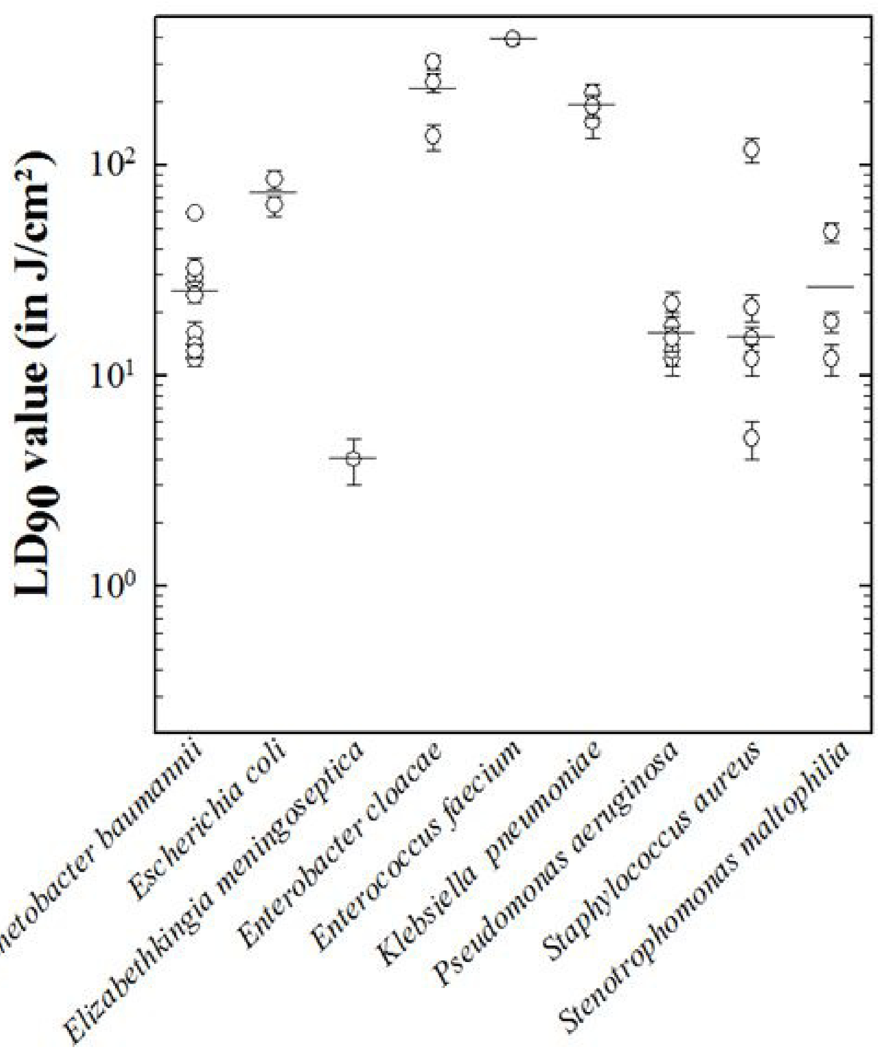

Figure 2: Comparison of blue light LD_{90} values between strains and species. Each individual circle represents the average LD_{90} for each strain ± standard deviations (*n* = 3). The average LD_{90} value for each species is shown by horizontal lines.

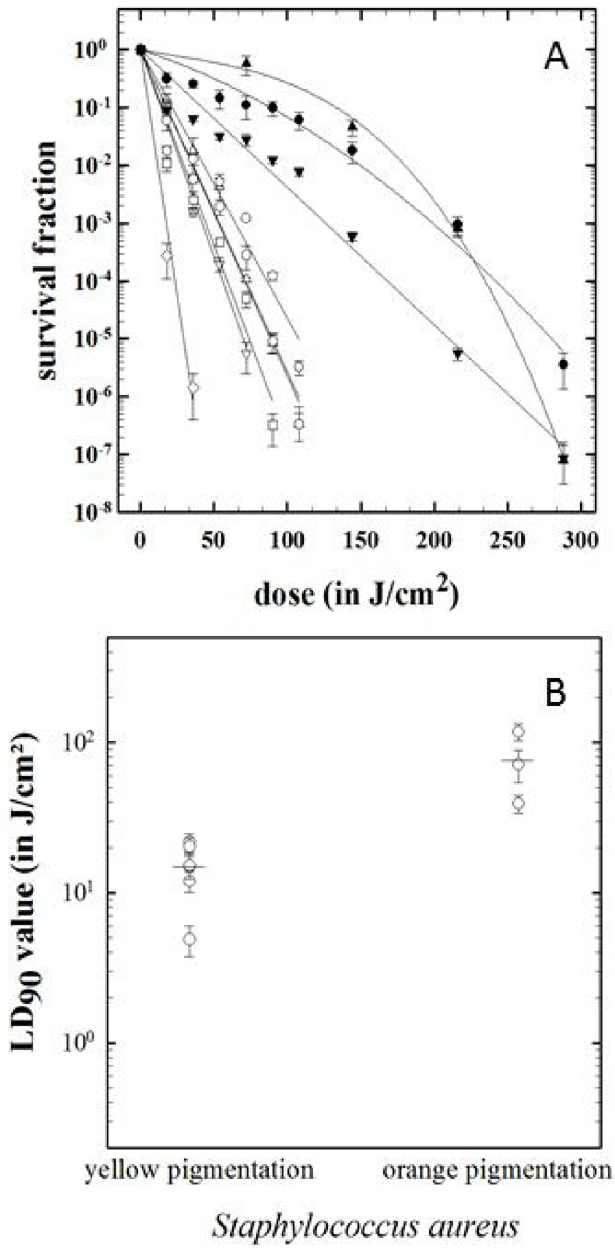

Figure 3. A: Correlation between survival of planktonic *S. aureus* strains following blue light exposure, and cell pigmentation, **B:** Comparison of blue light LD_{90} values between yellow and orange pigmented *S. aureus* strains.

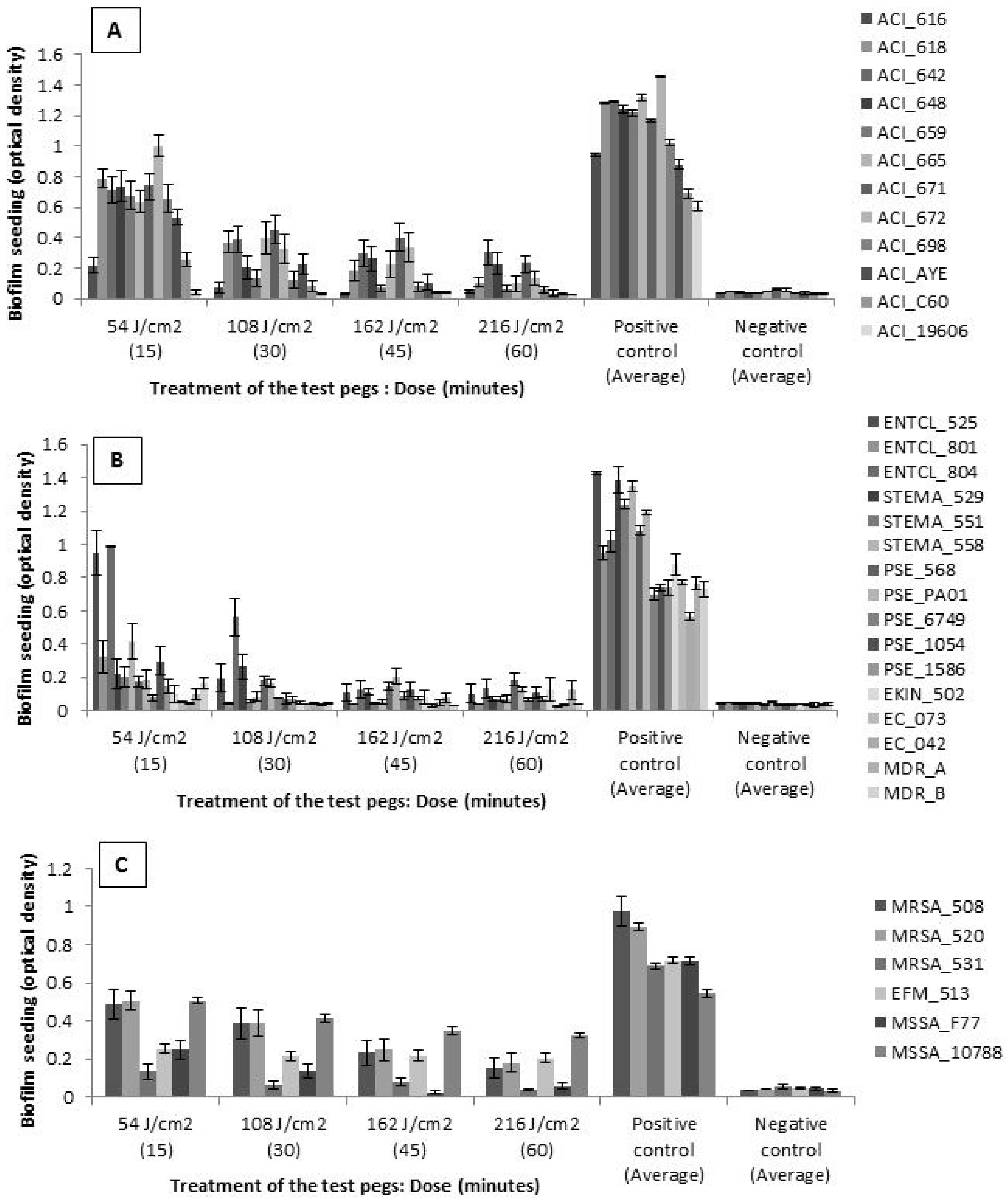

Figure 4, A-C: Graphs showing the biofilm seeding results for all isolates. Optical density on the y axis refers to the average biofilm seeding for the isolates tested after exposure to blue light at the range of durations tested (in minutes) on the x axis. Positive control: refers to the average biofilm seeding of the dark incubated, non BL exposed isolates. Negative control: refers to a negative (broth only) control. The error bars represent the standard error.

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Table 1: List of the clinical and control isolates used in this study.

Study Identifier	Organism	Description
ACI_616	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_618	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_642	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_648	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_659	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_665	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_671	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_672	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_698	*Acinetobacter baumannii*	QEHB clinical outbreak isolate
ACI_AYE	*Acinetobacter baumannii*	MPR Clinical Isolate (unique)
ACI_C60	*Acinetobacter baumannii*	NCTC_13424 (unique)
ACI_19606	*Acinetobacter baumannii*	ATCC_19606 (unique)
ENTCL_525	*Enterobacter cloacae* complex	QEHB clinical isolate
ENTCL_801	*Enterobacter cloacae* complex	QEHB clinical isolate
ENTCL_804	*Enterobacter cloacae* complex	QEHB clinical isolate
STEMA_529	*Stenotrophomonas maltophilia*	QEHB clinical isolate
STEMA_551	*Stenotrophomonas maltophilia*	QEHB clinical isolate
STEMA_558	*Stenotrophomonas maltophilia*	QEHB clinical isolate
PSE_568	*Pseudomonas aeruginosa*	QEHB clinical isolate
PSE_PA01	*Pseudomonas aeruginosa*	ATCC_15692
PSE_6749	*Pseudomonas aeruginosa*	NCTC_6749
PSE_1054	*Pseudomonas aeruginosa*	QEHB Clinical burn isolate
PSE_1586	*Pseudomonas aeruginosa*	QEHB Clinical burn isolate
EKIN_502	*Elizabethkingia meningoseptica*	QEHB clinical isolate
EC_073	*Escherichia coli*	EPEC CFT_073
EC_042	*Escherichia coli*	EAEC_042
MDR_A	CPE^ *Klebsiella pneumoniae* (NDM-1^{+} positive)	QEHB Clinical isolate
MDR_B	CRE* *Klebsiella pneumoniae* (ESBL positive with additional permeability changes)	QEHB Clinical isolate
MRSA_508	*Staphylococcus aureus*	QEHB Clinical isolate
MRSA_520	*Staphylococcus aureus*	QEHB Clinical isolate
MRSA_531	*Staphylococcus aureus*	QEHB Clinical isolate
MSSA_10788	*Staphylococcus aureus*	NCTC_10788
MSSA_F77	*Staphylococcus aureus*	NCTC_8532
EFM_513	*Enterococcus faecium*	QEHB Clinical isolate
MSSA_29213	*Staphylococcus aureus*	ATCC_29213
MSSA_10442	*Staphylococcus aureus*	NCTC_10442
MSSA_33807	*Staphylococcus aureus*	ATCC_33807
MSSA_4163	*Staphylococcus aureus*	NCTC_4163

Table 2: Antimicrobial effects of blue light on planktonic cells

Isolate	Exposure time (minutes)	Irradiance mW/cm^2	Dose J/cm^2	Log_{10} reduction	P-value	LD_{37} value + J/cm2		LD_{90} value + J/cm2	
ACI_616	30	60	108	7.06	0.006	21	± 2	27	± 2
ACI_618	30	60	108	5.78	0.007	55	± 4	59	± 3
ACI_642	30	60	108	6.73	0.006	9	± 1	16	± 2
ACI_648	30	60	108	6.14	0.007	21	± 2	29	± 3
ACI_659	30	60	108	6.55	0.006	8	± 1	16	± 2
ACI_665	30	60	108	6.14	0.006	7	± 1	12	± 1
ACI_671	30	60	108	6.34	0.006	25	± 2	32	± 4
ACI_672	30	60	108	6.22	0.006	16	± 2	24	± 2
ACI_698	30	60	108	6.39	0.008	9	± 1	14	± 2
ACI_AYE	30	60	108	6.70	0.006	10	± 1	16	± 2
ACI_C60	30	60	108	6.76	0.007	7	± 1	14	± 1
ACI_19606	30	60	108	6.81	0.006	7	± 1	13	± 1
ENTCL_525	100	60	360	6.76	0.006	113	± 12	136	± 19
ENTCL_801	180	60	648	6.61	0.009	212	± 20	246	± 25
ENTCL_804	160	60	576	6.24	0.007	258	± 18	306	± 24
STEMA_529	30	60	108	7.21	0.006	7	± 1	12	± 2
STEMA_551	30	60	108	2.97	0.006	26	± 3	48	± 5
STEMA_558	30	60	108	7.33	0.006	8	± 1	18	± 2
PSE_568	30	60	108	6.48	0.002	6	± 1	12	± 2
PSE_PA01	30	60	108	5.59	0.001	6	± 1	17	± 3
PSE_6749	30	60	108	6.55	0.009	7	± 1	13	±
PSE_1054	30	60	108	6.01	0.002	9	± 1	15	± 2
PSE_1586	30	60	108	6.07	0.002	13	± 2	22	± 2
EKIN_502	15	60	54	6.79	0.006	1	± 0.5	4	± 3
EC_073	30	60	108	4.71	0.006	56	± 4	64	± 7
EC_042	30	60	108	1.55	0.006	74	± 8	85	± 9
MDR_A	140	60	504	6.88	0.002	124	± 18	159	± 25
MDR_B	140	60	504	6.61	0.007	185	± 16	219	± 22
MRSA_508*	30	60	108	6.17	0.002	12	± 1	21	± 3
MRSA_520*	15	60	54	6.82	0.002	1	± 0.5	5	± 1
MRSA_531*	30	60	108	6.41	0.001	7	± 1	15	± 2
MSSA_10788 ^	80	60	288	7.07	0.001	99	± 12	118	± 15
MSSA_ F77*	30	60	108	6.76	0.006	3	± 1	12	± 2
EFM_513	180	60	648	1.86	0.007	277	± 16	393	± 20
Additional *S. aureus* isolates (for pigmentation investigation)									
ATCC_29213*	30	60	108	6.76	0.002	5	± 1	15	± 2
NCTC_10442*	30	60	108	6.69	0.002	8	± 1	20	± 2
ATCC_33807 ^	80	60	288	7.01	0.002	15	± 2	40	± 5
NCTC_4163 ^	80	60	288	6.07	0.003	38	± 5	71	± 6

Where * yellow pigmentation, ^ orange pigmentation, + LD37 and LD90 values are expressed ± standard deviation

Table 3: Average percentage change in biofilm seeding in isolates exposed to blue light compared to non-exposed dark incubated controls

Study Identifier	Average change in biofilm seeding (%) with blue light exposure			
	15 minutes ($54 J/cm^2$)	30 minutes ($108 J/cm^2$)	45 minutes ($162 J/cm^2$)	60 minutes ($216 J/cm^2$)
ACI_616	-75.9	-92.4	-96.5	-94.5
ACI_618	-39	-71.1	-85.6	-91.3
ACI_642	-45.6	-69.8	-77.3	-76.3
ACI_648	-43.7	-83	-78.2	-81.4
ACI_659	-47.1	-88.6	-94.1	-94.3
ACI_665	-53.9	-69.9	-82.5	-92
ACI_671	-37.4	-60.6	-65.7	-79.9
ACI_672	-31.1	-77.7	-76.5	-90.7
ACI_698	-36.7	-87.3	-92.2	-93.7
ACI_AYE	-41.9	-76.2	-86.7	-95.5
ACI_C60	-60.4	-89	-93.3	-94.8
ACI_19606	-93.5	-94.6	-93.2	-94.3
ENTCL_525	-34.9	-86.1	-92.2	-92.6
ENTCL_801	-61.3	-94.6	-95.6	-96.4
ENTCL_804	+18.7	-46.6	-88.2	-87.8
STEMA_529	-80.7	-81	-92.4	-95.1
STEMA_551	-84.5	-95.1	-96.2	-94
STEMA_558	-71	-93.3	-96.2	-94.7
PSE_568	-83.9	-82.8	-87.2	-81.8
PSE_PA01	-83.7	-86.2	-82.8	-89.5
PSE_6749	-88.9	-90.3	-87.1	-88.9
PS_1054	-58.3	-90.7	-83.2	-84.3
PSE_1586	-80.3	-92.0	-89.4	-88.8
EKIN_502	-85.8	-94.8	-91.6	-86.5
EC_073	-93.0	-94.6	-96.2	-96.2
EC_042	-92.1	-91.3	-92.1	-93.4
MDR_A	-87.4	-96.0	-89.2	-82.4
MDR_B	-75.3	-95.0	-95.8	-94.3
MRSA_508	-59.5	-58	-73.7	-83.3
MRSA_520	-44.5	-57.7	-73.2	-78.8
MRSA_531	-81.6	-91.2	-88	-93.7
MSSA_10788	-5.0 ^	-30.9	-36.3	-34.6
MSSA_F77	-67.8	-79.6	-96.4	-92.0
EFM_513	-66.3	-69.3	-68.2	-72.2

^ p value = 0.15. Shading denotes reductions of at least 80% in biofilm seeding compared to the positive control